

# Mutations of the gene encoding the protein kinase A type I- $\alpha$ regulatory subunit in patients with the Carney complex

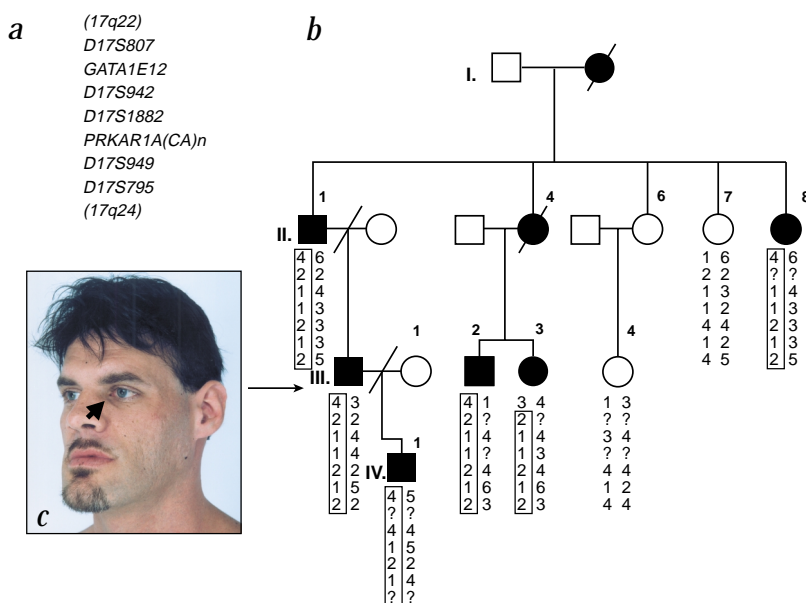
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Carney complex (CNC) is a multiple neoplasia syndrome characterized by spotty skin pigmentation, cardiac and other myxomas, endocrine tumours and psammomatous melanotic schwannomas<sup>1–5</sup>. CNC is inherited as an autosomal dominant trait and the genes responsible have been mapped to 2p16 and 17q22–24 (refs 6,7). Because of its similarities to the McCune-Albright syndrome<sup>5,8</sup> and other features, such as paradoxical responses to endocrine signals<sup>9</sup>, genes implicated in cyclic nucleotide-dependent signalling have been considered candidates for causing CNC (ref. 10). In CNC families mapping to 17q, we detected loss of heterozygosity (LOH) in the vicinity of the gene (*PRKARIA*) encoding protein kinase A regulatory subunit 1- $\alpha$  (R1 $\alpha$ ), including a polymorphic site within its 5' region. We subsequently identified three unrelated kindreds with an identical mutation in the coding region of *PRKARIA*. Analysis of additional cases revealed the same mutation in a sporadic case of CNC, and different mutations in three other families, including one with isolated inherited cardiac myxomas. Analysis of PKA activity in CNC tumours demonstrated a decreased basal activity, but an increase in cAMP-stimulated activity compared with non-CNC tumours. We conclude that germline mutations in *PRKARIA*, an apparent tumour-suppressor gene, are responsible for the CNC phenotype in a subset of patients with this disease. Genetic changes were identified at 17q22–24 in a pituitary tumour from a patient with CNC, belonging to a family in which the disease mapped to that region<sup>7</sup> (S.P., L.S.K. and C.A.S., manuscript submitted). This stimulated us to search for additional families mapping to 17q22–24 and for genetic alterations in their tumours. Polymorphic markers at 17q22–24 and proximal to *PRKARIA* (ref. 11) showed complete segregation with the disease in the CAR01 family (YC01 in ref. 7), a large pedigree with primary pigmented nodular adrenocortical disease (PPNAD) and other manifestations of CNC (ref. 12). The disease also segregated with markers from 17q in CAR20 (Fig. 1), a family that we have recently characterized (Table 1). We also identified a polymorphic sequence within the 5' region of *PRKARIA*

(*PRKARIA*(CA)n; ref. 13) that segregated in both families. We then searched for LOH in tumour specimens obtained from four members of the two families and the probands of other kindreds with CNC. All samples showed LOH of an 8-cM area of 17q22–24 around *PRKARIA* (Fig. 2a). The allele segregating with the disease was retained in all informative tumour samples (Fig. 2b).

To evaluate *PRKARIA* as a candidate for CNC, we used ESTs from *PRKARIA* to identify a bacterial artificial chromosome (BAC) that contained its genomic sequence (BAC 62\_F\_10). By comparing the cDNA (ref. 13) and genomic sequence, we delineated the intron-exon boundaries of the gene and found that what had been reported as a single exon 4 was actually split into two exons, which we termed 4A and 4B. We designed primer pairs for each exon and screened the gene using denaturing high performance liquid chromatography (DHPLC). This analysis showed heteroduplex formation in exon 4B in all affected members of families CAR01 and CAR20. Sequence analysis disclosed a 2-bp deletion at position 578 of *PRKARIA* in affected patients from both families (Fig. 3). The 578delTG mutation, which is located in exon 4B and corresponds to position 163 of the protein, leads to a frameshift and premature termination after 4 missense residues (Table 2). The families did not share 17q22–24 haplotypes of the disease-bearing allele, making it unlikely that they carried the same chromosome 17 (data not shown).

To confirm the identity of *PRKARIA* as the gene whose mutation causes CNC, we sought mutations of *PRKARIA* in other families and sporadic cases. One additional family (CAR108) and a patient



**Fig. 1** Pedigree and chromosome 17 genotyping of family CAR20. **a**, Polymorphic markers in the 17q22–24 region in members of family CAR20. The list of the markers is in chromosomal order according to the online databases. **b**, The boxed alleles indicate the affected chromosome in this family; there are no recombinations between this family and the 17q22–24 region. **c**, The proband of family CAR20 (CAR20.03), who presented with a cardiac myxoma. He also had acromegaly and facial pigmentation, including pigmented spots on his lips and pigmentation at the inner canthus of the eye (arrow), a clinical sign characteristic for CNC.

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**Table 1 • Clinical manifestations of individuals in family CAR20**

	Acromegaly	Cardiac myxoma	Lentiginos	Other <sup>a</sup>
II.1	+	—	+	LCCSCT
II.4	+	+	+	
II.6	—	—	—	
II.7	—	—	—	
II.8	+	+	+	
III.1 <sup>b</sup>	+	+	+	PMS, LCCSCT
III.2	+	+	+	
III.3	+	+	+	
III.4	—	—	—	
IV.1	—	—	+	

<sup>a</sup>LCCSCT, large-cell calcifying Sertoli cell tumour; PMS, psammomatous melanotic schwannoma. <sup>b</sup>Proband of the family.

with sporadic disease (CAR23.03) also carried the 578delTG mutation (Table 2). The mutation appears to have occurred *de novo* in CAR23.03, because it was not present in DNA purified from the peripheral blood of his parents (data not shown).

Screening additional patients with CNC revealed other mutations in *PRKARIA*. A GG→CT change at cDNA position 889, leading to premature termination after residue 204, was identified in kindred CAR13. A presumed splice-junction mutation at exon 8 was also found in family CAR25 (Table 2). Screening four other patients with CNC (from kindreds CAR19, CAR110, CAR06 and CAR09), whose tumours were examined for LOH (Fig. 2a), has not yet revealed mutations in the coding regions of the gene. Probands from other families mapping to chromosome 2p16 or elsewhere<sup>6,14</sup> did not have detectable alterations in *PRKARIA* (data not shown). We also searched for *PRKARIA* mutations in patients with either isolated myxomas or PPNAD. One family that had cardiac myxomas and no other CNC manifestations<sup>15</sup> had a 4-bp deletion in exon 5 of *PRKARIA*, leading to frameshift and premature termination after 26 missense residues (family MYX01; Table 2). None of the three patients with sporadic PPNAD were found to carry a mutation in the coding sequences of *PRKARIA*.

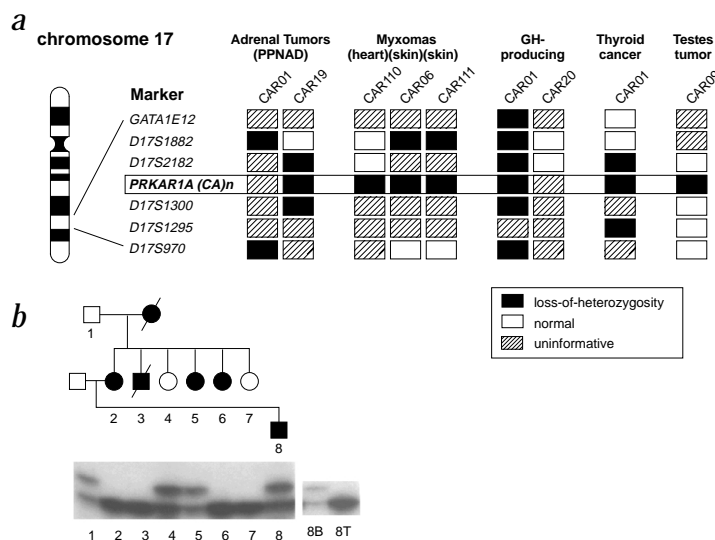
To determine the functional consequences of these mutations, we measured the cAMP responsiveness and PKA activity ratio in tumour cell extracts from families CAR01, CAR20 and CAR25 and compared them with tumour extracts from non-CNC patients (Fig. 4a,b). Measurements of non-stimulated PKA activity were not different between CNC tumours and non-CNC tumours (Fig. 4a, left). Addition of cAMP led to a stimulation in kinase activity in CNC tumours (kinase activity at baseline versus following stimulation with cAMP,  $P < 0.001$ ; Fig. 4a). This response was PKA-specific, as demonstrated by its inhibition by protein kinase inhibitor (PKI), a specific PKA-inhibitor<sup>16</sup> (kinase activity in response to cAMP versus cAMP and PKI,  $P < 0.001$ ), and it was not different between tumours with the 578delTG genotype

(2 tumours from family CAR01 and 2 from family CAR20) and those with a different *PRKARIA* mutation (2 tumours from CAR25). Both the stimulation of kinase activity with cAMP and the inhibition of that stimulation by PKI were greater in CNC tumours than in those in the control samples ( $P < 0.05$ ). PKA activity ratio, a measure of how much PKA is in its active form<sup>16</sup> (free/basal PKA), was decreased in CNC tumours compared with the control samples ( $P < 0.001$ ), as expected from  $R\alpha$  inactivating mutations (Fig. 4b).

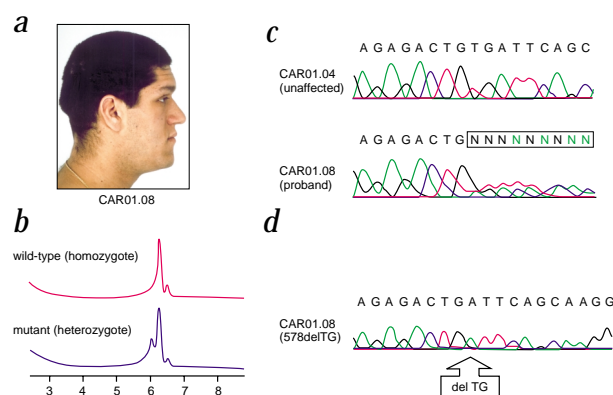
Western-blot analysis of cellular proteins from a primary cell line from the CAR20.03 pituitary tumour revealed the presence of full-length *PRKARIA* in the cells (Fig. 4c, lane 1). No truncated forms of the protein were observed. Control experiments with bacterially expressed GST fusion proteins confirmed the ability of this antibody to detect the truncated protein (Fig. 4c, lanes 2–4). Moreover, RT-PCR of the *PRKARIA* message from this cell line detected only wild-type sequence (data not shown), suggesting that the mutant mRNA may be unstable in the cells. Mutant mRNA was also not detected in another cell line that was established from a member of family CAR25 bearing a splice site mutation at the 5' splice-site of intron 8 (data not shown). This observation is consistent with the cellular elimination of mutant mRNAs, as observed in other settings<sup>17</sup>.

We have identified mutations in *PRKARIA* in several families with CNC and patients with the sporadic form of the disease. Three unrelated families and one sporadic case shared the same 2-bp deletion in exon 4B of *PRKARIA*, suggesting that this site may contain a hot spot for mutation. The mechanism responsible for mutagenesis at this site remains unclear; DNA polymerase stuttering may have a role because of the TGTG pattern present at the deletion site<sup>18</sup>, although other mechanisms cannot be excluded. A defective cyclic-nucleotide-dependent pathway has been considered a candidate mechanism for the features of CNC (refs 5,6,10). One of the first candidate gene analyses for CNC was screening for *GNAS1* (the gene responsible for McCune-Albright syndrome; ref. 8) mutations; this study produced negative results<sup>10</sup>. It was then suggested that the defect in CNC was downstream from cAMP activation. The PKA holoenzyme, a critical component in cAMP-dependent signalling, was thus a likely candidate for the identification of mutations in patients with CNC. In addition to mutations inactivating  $R\alpha$ , we identified LOH for *PRKARIA* in CNC tumours. The PKA activity ratio was also decreased in CNC tumours. These data are consistent with the hypothesis that normal  $R\alpha$  functions as a tumour suppressor in tissues affected by CNC, perhaps by maintaining normal type-I PKA activity. In the absence of normal  $R\alpha$ , there appears to be an increased stimula-

**Fig. 2** Loss-of-heterozygosity analysis of chromosome 17 near *PRKARIA*. **a**, LOH analysis was performed using paired samples of blood and tumour DNA from probands of the indicated families and markers located 8 cM around *PRKARIA*, including *PRKARIA*(CA)<sub>n</sub>, a dinucleotide repeat from the 5'-region of the gene. The tumours were adrenal (PPNAD) from individual CAR01.06 and the proband of family CAR19; myxomas from kindreds CAR110, CAR06 and CAR111; growth-hormone-producing pituitary adenomas from CAR01.08 and CAR20.03; a thyroid follicular carcinoma from individual CAR01.05; and a testicular tumour (large-cell calcifying Sertoli cell tumour, LCCSCT) from kindred CAR09. All informative loci demonstrated LOH at the *PRKARIA* locus, indicated by the interval between the bars. LCCSCT, large-cell calcifying Sertoli cell tumour; PPNAD, primary pigmented nodular adrenocortical disease. **b**, The pedigree of family CAR01. Marker GATA1E12 has two alleles: the upper allele is derived from the unaffected father. LOH analysis of the pituitary tumour of CAR01.08 demonstrates loss of the upper allele in the tumour DNA (8T). 8B, peripheral blood lymphocytes DNA; 8T, pituitary tumour DNA.



**Fig. 3** Detection of a frameshift mutation in *CAR01* in *PRKAR1A* exon 4B. **a**, Typical acromegaly and some facial pigmentation in individual *CAR01.08*. **b**, Members of family *CAR01* were tested for the presence of heteroduplexes in exon 4B using denaturing HPLC analysis. There is an alteration in peak shape in *CAR01.08* indicative of heteroduplex formation (heterozygosity), whereas the tracing from an unaffected individual is shown above. **c**, Sequence traces from a homozygote (*CAR01.04*) and a heterozygote (*CAR01.08*) are shown, indicating the presence of a sequence alteration (boxed) in the proband's genomic DNA. **d**, Heteroduplex DNA with the identified change was subsequently cloned for clarification of the sequencing defect; the colonies were selected and sequenced, revealing the presence of a 2-bp deletion in the mutant form of the sequence (578delTG) shown by the arrow.



tion by cAMP, perhaps mediated by overexpression of genes encoding the other regulatory subunits of the PKA complex. This type of compensatory response has been demonstrated in animal models lacking one of the subunits of the PKA holoenzyme<sup>19,20</sup>. Furthermore, the ratios of type-I PKA to type-II PKA can change dramatically during cell development, differentiation and transformation<sup>16,21</sup>, indicating that the PKA complex has substantial flexibility in its mediation of cAMP signalling. Other possible mechanisms for tumorigenesis in CNC, which would apply especially in the cases of point mutations of *PRKAR1A*, include an uninhibited catalytic subunit of the PKA complex, which by itself when mutated leads to unregulated PKA activity<sup>22</sup> or a reduced turnover of the cAMP molecule<sup>23</sup>.

## Methods

**Patients and patient samples.** The institutional review boards of NICHD, NIH and the Mayo Clinic approved the contact of the families and the participation of the family members in the NICHD protocol 95-CH-059 after obtaining informed consent. We classified patients with CNC as 'affected' according to described criteria<sup>3,6</sup>. Blood and tissue samples were collected from patients belonging to described families<sup>6,12,14</sup> and sporadic cases. The clinical profile of the patients from family *CAR01* (YC01) has been reported<sup>12</sup>; those of family *CAR20* are described in Table 1.

**Microsatellite and loss-of-heterozygosity analysis.** We investigated family *CAR20* for segregation of CNC with the polymorphic 17q22–24 markers as described<sup>6,24</sup>. For LOH analysis, DNA from CNC tumours was analysed along with a paired DNA sample from the patient's peripheral blood. The microsatellite alterations seen in the tumours were classified as described<sup>24,25</sup>. Uninformative samples were those that demonstrated either homozygosity or microsatellite length instability.

***PRKAR1A* genomic structure and primer design.** The cDNA structure of human *PRKAR1A* has been reported<sup>13</sup>. BLAST analysis of this sequence revealed that exons 3–10 were contained in the RPCI BAC 62\_F\_10, although the intron-exon structure predicted from the genomic sequence was different from that described (data not shown). We generated primers to amplify these exons from the genomic sequence using the Whitehead Institute's World-Wide Web-based program Primer3 (<http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi>). The 5' end of the gene is contained in GenBank Y07641, and the same program was used to select primers to amplify exons 1A and 1B, as well as an apparent CA repeat in the

promoter of the gene (*PRKAR1A*(CA)<sub>n</sub>). Sequences of the primers used here are available on request.

**Denaturing HPLC analysis.** DNA samples from patients and unaffected family members were PCR amplified in 30-μl volumes using AmpliTaq Gold (Perkin-Elmer). We injected samples (10 μl) into a DHPLC instrument (HELIX, Varian) at column temperatures recommended by the DHPLC Melt program (<http://insertion.stanford.edu/melt.html>), as described<sup>26,27</sup>.

**Analysis of mutations.** In heteroduplex samples, the remaining 20 μl was purified and sequenced using BigDye Terminators (Perkin-Elmer) on an ABI 377 fluorescent sequencer. The sequence traces were analysed using either Sequencher (Genecodes) or Vector NTI (Informax). In cases where the sequence change appeared to be a frameshift, PCR samples were subject to TOPO-TA cloning (Invitrogen) and sequencing of the plasmids contained in individual colonies.

**PKA activity.** We measured PKA activity as described<sup>16,28</sup> in cell extracts from 6 CNC tumours (3 PPNAD, 1 ovarian tumour and 2 schwannomas) and 4 non-CNC tumours (3 adrenocortical adenomas and 1 meningioma). All of the CNC tumours were from individuals known to contain mutations in *PRKAR1A*. Activity was compared separately in the tumours from steroid-producing tissues (3 PPNAD and 1 ovarian tumour from CNC patients versus 3 adrenal adenomas from non-CNC patients) and the CNS tumours (2 schwannomas from CNC tumours versus 1 meningioma from a non-CNC patient).

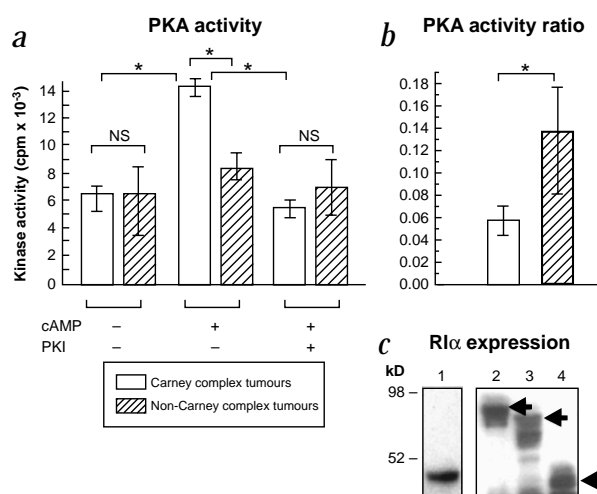
The PKA activity ratio was calculated as described<sup>16</sup>. All determinations of PKA activity were done twice for each tumour and an average value was calculated for each experiment. Data from all tumours were compared with the STATISTICA software (StatSoft) using *t*-test for individual comparisons. A *P* value of less than 0.05 was considered significant.

**Preparation of proteins and western-blot analysis.** Primary cultured cells from the pituitary tumour of individual *CAR20.03* were maintained in RPMI-1640 supplemented with 15% fetal bovine serum. After pelleting and washing with phosphate-buffered saline (PBS), the cells were resuspended in 3 volumes of M-PER reagent (Pierce) supplemented with complete protease inhibitor cocktail (Roche Biochemicals). We determined protein concentrations by the BCA assay kit (Pierce) and

**Table 2 • Mutations in *PRKAR1A* in families with CNC**

Kindred	Diagnosis	Mutation	Effect on R1α
CAR01	CNC		
CAR20	CNC	578 delTG (exon 4B)	frameshift after codon 163; stop codon after 4 missense residues; truncation before the cAMP binding regions
CAR108	CNC		
CAR23.03 (sporadic)	CNC		
CAR13	CNC	889 GG>CT (exon 8)	missense codon 262, followed by nonsense codon; truncation at N terminus of the second cAMP binding domain
CAR25	CNC	exon8 IVS+3 A>G	mutation of 5' splice site of intron 8; effect on protein uncharacterized
MYX01	Inherited cardiac myxoma	617delTTAT (exon 5)	frameshift after residue 204; stop codon after 26 missense residues; the mutation would also abolish the second cAMP binding domain





**Fig. 4** PKA activity and expression of PRKAR1A in CNC tumours and cell lines. **a**, Total PKA activity was not different between four CNC steroid-producing tumours from families CAR01, CAR20 and CAR25 and three control tumours from non-CNC patients at baseline. PKA activity increased in response to cAMP in CNC tumours, and that increase was blocked by PKI (for both  $P < 0.001$ ). These responses were greater in CNC tumours than the corresponding changes in non-CNC tumours ( $P < 0.05$ ); in addition, the peak kinase activity value in response to cAMP was higher in CNC tumours versus non-CNC tumours ( $P < 0.001$ ). All error bars represent standard error of the mean; NS, non-significant; \*, significant at  $P < 0.05$ ; +, -, presence or absence of cAMP or PKI, respectively. **b**, PKA activity ratio calculated as the ratio between free (basal) PKA and total PKA was lower in six CNC tumours bearing inactivating PRKAR1A mutations than that in four non-CNC samples ( $P < 0.001$ ). **c**, Western-blot analysis of protein lysates using a PRKAR1A-specific antibody. Lane 1, total cellular proteins prepared from a CNC cell line from patient CAR20.03; lanes 2-4, lysates from bacteria containing expression constructs containing GST-PRKAR1A expression plasmids; lane 2, PRKAR1A nt 74-1,228 (full-length, expected size 72 kD); lane 3, PRKAR1A nt 74-871 (exon 8 truncation, expected size 59 kD); lane 4, PRKAR1A nt 74-577 (exon 4B truncation, expected size 48 kD). Size markers are indicated at left. Arrowheads denote expected size of fusion proteins. Bands of smaller size most likely represent degradation products.

resolved protein (20 µg) in a 4-12% NuPage gel in MES buffer (Invitrogen) before transfer to PVDF membranes. Western blots were performed using the Western Breeze kit (Invitrogen). We used monoclonal antibodies specific for PRKAR1A as specified by the manufacturer (BD Transduction Laboratories).

**Generation and western-blot analysis of GST-PRKAR1A fusion proteins.** We designed PCR primers containing *attB* sequences using VectorNTI software (Informax) to amplify fragments of the PRKAR1A cDNA corresponding to nt 74-577, 74-871 or 74-1,228. These fragments were PCR-generated from normal adrenal gland mRNA and cloned into pDONR201. After sequence verification, the inserts were transferred using Gateway Technology (Life Technologies) into pDEST15, which contains an amino-terminal GST coding region under the control of the T7 promoter. The constructs were transformed into BL21(SI) cells and proteins prepared after overnight growth in LB broth with or without sodium chloride (0.17 M). Bacteria (1.5 ml) were pelleted and resuspended in B-PER reagent (300 µl; Pierce). Bacterial extract (18 µl) was resolved on a NuPage gel and western blotted as above. The anti-GST antibody (Pierce) was used at a dilution of 1:1,000.

GenBank accession number. BAC 62\_F\_10, AC005799.

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